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(57) Abstract

Neurofibromatosis type 1 disorders result in abnormal levels of midkine protein and/or the mRNA sequence encoding the midkine protein in the skin and/or blood serum of individuals having the disorder. Thus, test for abnormal levels of midkine protein and/or the mRNA sequence that encodes the midkine protein can be used to diagnose a neurofibromatosis type 1 disorder.

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METHOD OF DETECTING AND TREATING NEUROFIBROMATOSIS TYPE 1

This application claims the benefit of U.S. Provisional Application No. 60/109,404, filed November 20, 1998, which is herein incorporated by reference hereto.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to biochemical methods of detecting and treating cell growth disorders, particularly those cell growth disorders having a genetic cause, including disorders associated with loss of tumor suppressor function. More particularly, the present invention relates to detection and treatment of neurofibromatosis type 1 disorders.

2. State of the Art

Many cell growth disorders are known in the medical literature. Among the most important of these disorders are those resulting in neoplasias, including both benign and cancerous tumors. The health and financial costs to not only individuals, but to society as a whole as well, of neoplastic cell growth disorders are tremendous. Therefore, the medical and scientific communities are researching the causes of these disorders in an effort to understand the ultimate causes and provide effective, convenient, and affordable detection and treatment systems.

For example, neurofibromatosis type 1 (NF1) is a common autosomal dominant disorder (incidence 1:3500) characterized by abnormalities of neural crest derivatives such as Schwann cells and melanocytes (Gutmann and Collins, 1995). These abnormalities are often cutaneous in nature, and are manifested as multiple dermal neurofibromas, café-au-lait macules, abnormal freckling, Lisch nodules, and hyperpigmentation (Gutmann and Collins, 1995). The heterozygous germ-line loss of *NF1*, and subsequent reduction of the tumor suppressor neurofibromin, is thought to be the critical event in the pathogenesis of NF1. Additionally, secondary genetic and epigenetic events such as growth factor dysregulation may be involved (DeClue et al, 1991; Gutmann et al., 1991; Bernards, 1995).

The present inventors have previously determined that loss of both alleles of *NF1* in murine Schwann cells results in the upregulation of the growth factor midkine

(MK). MK is a secreted 13 kD heparin-binding polypeptide that is highly expressed during mouse embryogenesis in the neuroectoderm and regions of secondary epithelial-mesenchymal induction (Muramatsu et al. 1993; Mitsiadis et al, 1995). In the adult, residual MK expression is detected in kidney, intestine, and cerebellum (Kadomatsu et al. 1990; Nakamoto et al. 1992; Muramatsu et al. 1993; Matsumoto et al., 1994). Overexpression of MK in the adult has been found to be associated with a variety of tumors (Garver et al., 1994; Nakagawara et al., 1995; O'Brien et al., 1996), and is thought to promote angiogensis and tumorigenesis (Kadomatsu et al., 1997; Choudhuri et al., 1997).

In addition, the dysregulation of growth factors has previously been investigated in the pathogenesis of NF1. Functional studies on neurofibromin-deficient neurons indicate that neurotrophic factors are directly regulated by neurofibromin, as evidenced by the survival of sensory embryonic neurons isolated from homozygous NF1 knockout mice in the absence of neurotrophic factors (Vogel et al., 1995). Furthermore, a number of growth factors and growth factor receptors that are overexpressed in neurofibromas have been reported. These include upregulation of nerve growth factor (NGF) (Fabricant and Todaro, 1981) neuroblastoma and glial growth factors (Asai et al., 1991), ciliary neurotrophic factor (Asai et al., 1991), insulin-like growth factor 2 (IGF-2) (Hansson et al., 1988), hepatocyte growth factor (Krasnoselsky et al., 1994; Rao et al., 1997), stem cell factor (Ryan et al., 1994), as well as receptors for nerve growth factor (Sonnenfeld et al., 1986), platelet derived growth factor (PDGF) (Kadono et al., 1994), transforming growth factor-β (TGF-β1) (Kadono et al., 1994), and c-kit (Hirota et al., 1993; Ryan et al., 1994). Schwann cells, fibroblasts, and neurons have all been examined for their role in the aberrant expression of tumor growth factors.

It has previously been demonstrated that normal human keratinocytes express neurofibromin (Malhotra and Ratner, 1994; Hermonen et al., 1995), but the significance of this in the symptoms of NF1 has not been clarified. Furthermore, it is currently unknown how the heterozygous loss of NF1 affects neurofibromin levels in keratinocytes.

Detection of NF1 has been limited to costly and time- and labor-intensive assays. For example, U.S. Patent No. 5,227,292 to White et al. discloses methods for the detection of defective neurofibromatosis type 1 (NF1) genes. It further discloses

methods of treatment of humans having a defective NF1 gene, and methods for detection of tumors caused by a defective NF1 gene. The methods of screening humans to identify those at risk of developing neurofibromatosis use a DNA diagnostic test in which the nucleic acid of a human is analyzed. The screening procedure according to White et al. includes (1) testing a nucleic acid sample of a patient for large deletions in the NF1 gene locus and, if large deletions are not detected, (2) testing a nucleic acid sample of a patient for small deletions or point mutations in the NF1 gene locus. Thus, because of the large size and multiple sites of mutation of the NF1 gene, direct molecular analysis of the gene is not technically feasible on a cost-effective basis.

Furthermore, U.S. Patent No. 4,582,787 to Frankel discloses a method for testing a patient to determine a predisposition to lung cancer and neurofibromatosis. The test relies on transformation of cells with Kirsten murine sarcoma virus and does not indicate a role for the MK protein in the disorder.

Also, detection methods for the MK protein have been developed. For example, Muramatsu et al. (J. Biochem, **119**:1171-1175, 1996) discloses a highly sensitive enzyme-linked immunoassay for Midkine (MK) which involves affinity-purified anti-MK antibodies, their biotinylated form, and avidin-beta-galactosidase. The method allows the detection of human and mouse MK in the range of 50 pg to 10 ng. Employing this method, Muramatsu et al. detected MK in developing mouse brains and determined the level. The assay can also be applied to serum samples to detect MK in hepatocellular carcinomas.

However, none of these references disclose methods for diagnosis and/or treatment of NF1 using Midkine growth factor or its corresponding nucleic acids as a marker for NF1. This may be due, at least in part, to the fact that it is not known whether overexpression of MK in NF1 patients is a result or a cause of tumorigenesis.

SUMMARY OF THE INVENTION

Individuals with NF1 but no family history manifest symptoms early in childhood but often do not reach diagnostic criteria until puberty. Large gene size, multiple mutation sites, and multiple pseudogenes have made direct molecular analysis of the NF1 gene technically difficult and have limited the usefulness and practicality of gene-based diagnostic techniques and treatment methods. Therefore, a

convenient, rapid, reliable, and inexpensive method for detecting, diagnosing, and treating this disorder is needed.

The present invention provides a method of diagnosing NF1 by detecting increased levels of the MK protein or its associated mRNA in the skin or blood serum of an individual, such as a patient. The diagnostic method of the invention is achieved by the discoveries that increased level of MK mRNA and protein in the skin of individuals and increased levels of MK protein in the blood serum of individuals are independent of the presence of a tumor, but indicative of an NF1 or pre-NF1 state in the individual.

Therefore, the present invention provides a method of using the MK protein and mRNA as a marker for NF1 and for the predilection to develop the disorder.

The present invention also provides an early detection method for the NF1 disorder using the MK mRNA and/or protein as a marker.

The present invention further provides a molecular diagnosis method for NF1 disorders. The invention avoids the technical difficulties of molecular analysis at the DNA level by detecting abnormal levels of MK mRNA and protein.

The present invention thus also provides a method of treatment of NF1 by instituting a treatment regimen for NF1 prior to the detection of a neurofibroma or other symptom of NF1. This present treatment method is enabled by the novel detection method of the invention, which allows detection of the NF1 disorder in individuals at an early stage in the development of the disorder, independent of a neurofibroma or other clinically conclusive symptoms, thus allowing a greater probability of successful treatment than methods initiated after development of the disorder. Accordingly, the present invention provides improved clinical management of the NF1 disorder.

The present discovery provides the first demonstration that the skin of NF1 patients is a source of aberrant tumorigenic and angiogenic MK growth factor expression. Because the method of diagnosing NF1 of the present invention can be routinely performed, and because the skin is easily accessible to biopsy, such as a punch biopsy, this discovery provides an efficient and cost-effective method of diagnosis of NF1.

The present discovery also provides the first demonstration that the blood serum of NF1 patients is a source of aberrant tumorigenic and angiogenic MK growth

factor. Because the method of diagnosing NF1 of the present invention can be routinely performed, and because a patient's blood is easily accessible, this discovery provides another efficient and cost-effective method of diagnosis of NF1.

The present invention provides a method for diagnosing a neurofibromatosis type 1 disorder in an individual, comprising obtaining a skin or blood serum sample from the individual; detecting the level of midkine protein and/or the mRNA sequence encoding the midkine protein present in the sample; and comparing the detected level of midkine protein and/or mRNA in the sample to the level of midkine protein and/or mRNA present in at least one corresponding skin or blood serum sample of at least one individual that does not have a neurofibromatosis type 1 disorder. In the method, a higher level of midkine protein and/or the mRNA sequence encoding the midkine protein in the sample of the individual being tested as compared to in the at least one corresponding sample of the at least one individual that does not have a neurofibromatosis type 1 disorder indicates the likelihood of a neurofibromatosis type 1 disorder in the individual being tested. In conducting these tests, it is, of course, not necessary to test a new sample of at least one individual that does not have a neurofibromatosis type 1 disorder each time a new individual is being tested. Instead, the comparison may be based on previously established levels in individuals that do not have a neurofibromatosis type 1 disorder.

In an embodiment of the invention, the method for diagnosing a neurofibromatosis type 1 disorder in an individual comprises obtaining a skin or blood serum sample from the individual; detecting the level of midkine protein and/or mRNA encoding the midkine protein in the sample; and determining whether the detected level of midkine protein and/or mRNA encoding the midkine protein is higher than a predetermined amount, the predetermined amount being greater than the concentration of midkine protein and/or mRNA encoding the midkine protein in the skin or blood serum of individual(s) that do not have a neurofibromatosis type 1 disorder, wherein the presence of an amount higher than the predetermined amount indicates the likelihood of a neurofibromatosis type 1 disorder.

In a further embodiment, the present invention is directed to a method for diagnosing the presence of a tumor in an individual, particularly tumors of the nervous system. The method comprises obtaining a blood serum sample from the individual; detecting the level of midkine protein in the sample; and comparing the detected level

of midkine protein to the level of midkine protein present in at least one blood serum sample of at least one individual that does not have a tumor, wherein a higher level of midkine protein in the sample of the individual being tested as compared to in the at least one blood serum sample of the at least one individual that does not have a tumor indicates the likelihood of a tumor in the individual being tested.

In a final embodiment, the present invention provides a method of culturing tissue culture cells by adding purified MK protein to the tissue culture medium.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows that MK is expressed in NF1 but not normal skin.

Scale bar = 200 μm for panels a-g; scale bar = 400 μm for panel h

White asterisks: examples of MK-positive regions of skin.

Black asterisks: examples of MK-negative regions of skin.

(a) *In situ* hybridization (ISH) of MK antisense digoxigenin-labeled riboprobe to skin with a dermal neurofibroma from NF1 patients (n=4). Arrows: melanocytes in the basal layer.

(b) ISH of MK antisense to skin with a dermal neurofibroma from a non-NF1 patient (n=1).

(c) ISH of MK antisense to skin without phenotypic abnormality from an NF1 patient (n=1).

(d) ISH of MK antisense to skin without phenotypic abnormality from a healthy subject (n=4).

(e) MK immunohistochemistry of skin without phenotypic abnormality from an NF1 patient (n=3).

(f) MK immunohistochemistry of skin without phenotypic abnormality from a healthy subject (n=3).

(g) ISH of FGF-2 antisense to a dermal neurofibroma from an NF1 patient (n=3). Arrow: blood vessel.

(h) ISH of FGF-2 antisense digoxigenin-labeled riboprobe to normal skin from a healthy subject (n=3).

Figure 2 demonstrates the increased levels of MK protein in the blood serum of individuals with NF1 as compared to individuals that do not have NF1.

Figure 3 shows that the mean percent growth of cells in the presence of MK is above the level of growth of control cells without MK.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention provides a molecular detection method that provides a means for detecting, diagnosing, and treating NF1. In embodiments, the molecular detection method detects abnormal levels of MK protein in skin cells of an individual. In embodiments, the molecular detection method detects abnormal levels of mRNA encoding the MK protein. In embodiments, the molecular detection method uses the MK protein and/or mRNA as a marker for NF1. In embodiments, the molecular detection method provides the initial steps in a method of treatment of NF1.

In an embodiment of the invention, a neurofibromatosis type 1 disorder is diagnosed in an individual by a method comprising obtaining a skin sample from the individual; and detecting the presence of midkine protein and/or the mRNA sequence encoding the midkine protein in the skin sample, the presence of the midkine protein and/or the mRNA sequence in the sample indicating the likelihood of a neurofibromatosis type 1 disorder.

In an embodiment of the present invention, a method of detecting the MK growth factor protein in skin cells of an animal, especially a human, is provided. The skin cells can be obtained from any individual, including those suffering from NF1, those suspected of having NF1, those at high risk or having a predisposition for NF1, such as a person with a known family history of NF1, and even those with no apparent signs or predisposition for NF1. The novel method relies on the discovery that there is abnormal protein expression in the skin of NF1 patients.

The method can include obtaining a sample of skin from the individual, optionally isolating protein from the skin, and detecting the level of MK growth factor protein and comparing the level with the level of MK protein in skin obtained from a normal (non-NF1) individual by the same technique, to determine if the MK protein is present in abnormal levels. This method not only provides a qualitative determination of the presence of MK at normal or abnormal levels, but also a semi-quantitative and quantitative method of determining the level of expression of the MK protein in the skin cells of the individual and a direct indication of the presence of the NF1 disorder in an individual.

In a particular embodiment of the invention, the presence of at least 10 ng of the midkine protein per one mm^3 of skin indicates the likelihood of a neurofibromatosis type 1 disorder.

Techniques for isolating and culturing skin cells are well known to the skilled artisan. Any suitable technique can be used according to the present invention.

The optional method for isolation of the MK protein can include any protein purification method steps known to the skilled artisan that result in isolation and/or purification of the MK protein and can be performed on any skin cells.

The method of detecting the MK protein can be any method for protein detection known to the skilled artisan, including staining for protein with a non-specific stain, such as coomassie blue staining of an acrylamide gel, specifically detecting the MK protein by using antibodies specific for the MK protein (e.g., Western blotting or other ELISA techniques), or determining absorbance at a wavelength of approximately 280 nm (UV absorption).

The method can also include performing an immunohistochemistry assay on skin biopsies or tissue samples. The immunohistochemistry assay can be any assay known to the skilled artisan, such as an immunofluorescence assay. A preferred method includes the following steps. Paraffin-embedded neurofibroma and/or normal skin tissue sections are incubated overnight at 55°C, microwaved at full power for 10 min (or a sufficient time to melt the remaining paraffin), cooled for 5 min at room temperature (approximately 22-25°C) and treated with xylene for 10 min. After two washes in 100% ethanol for 5 min each, the slides are rehydrated and washed in H_2O and phosphate-buffered saline (PBS) for 1 min each. Slides are then treated in PBS/1% (v/v) acetic acid at 80°C for 10 min. The slides are then cooled to room temperature and washed twice in PBS. The tissue is then blocked with 5% goat normal serum in PBS/2% bovine serum albumin (BSA) for 30 min. For immunohistochemistry, a polyclonal anti-MK antibody, such as rabbit anti-MK, is used at an appropriate dilution, such as approximately 1:1000. Antibody is applied overnight at 4°C, and slides are washed 3 times in PBS/Tween. A biotinylated goat anti-rabbit secondary antibody at an appropriate dilution, such as 1:500 is then applied for 30 min at room temperature, followed by 3 washes in PBS/Tween. For detection and visualization, the ABC system (Vector, Burlingame, CA) can be used according to the manufacturer's protocol with 0.1 mg/ml DAB as substrate. Slides are counterstained with hematoxylin for 1 min, dehydrated in ethanol and xylene, and coverslipped using a non-aqueous mounting medium. The presence and relative level

of MK protein in the control (non-NF1) and patient's sample are then determined using fluorescence microscopy.

In an embodiment, the present invention provides a method of detecting mRNA encoding the MK growth factor protein in skin cells of an animal, especially a human. The skin cells can be obtained from any individual, including those suffering from NF1, those suspected of having NF1, those at high risk or having a predisposition for NF1, and even those with no apparent signs or predisposition for NF1. The novel method relies on the discovery that there is abnormal mRNA expression in the skin of NF1 patients.

The method of detecting MK-specific mRNA can include obtaining a sample of skin from the individual, optionally isolating mRNA from the skin, and detecting the level of MK growth factor protein-specific mRNA and comparing the level with the level of MK mRNA in skin obtained from a normal (non-NF1) individual by the same technique, to determine if the MK mRNA is present in abnormal levels. This method not only provides a qualitative determination of the presence of MK mRNA at normal or abnormal levels, but also a semi-quantitative and quantitative method of determining the level of expression of the MK mRNA in the skin cells of the individual. The levels of mRNA in the cells can be correlated to the levels of MK protein in the cells and is a direct indicator of the presence of the NF1 disorder.

Techniques for isolating and culturing skin cells are well known to the skilled artisan. Any suitable technique can be used according to the present invention.

Techniques for isolating and detecting mRNA are well known to the skilled artisan. Any suitable technique can be used according to the present invention.

The method of the invention can include isolating total cellular RNA from human skin samples obtained from a patient using standard RNA isolation techniques. For the detection of mRNA, Northern blots are used. For Northern blots, 20 μg of total RNA from each tissue sample is separated in an agarose gel and transferred onto nitrocellulose. Blots are hybridized with [^{32}P]-labeled full length human cDNA probes for MK. A [^{32}P]-labeled 0.5 kb cDNA fragment of the glucose-6-phosphate dehydrogenase (GAPDH) housekeeping gene is used for control hybridizations. Hybridization is carried out in buffer containing 50% formamide at 42°C. Blots are subsequently washed in 0.1xSSC, 0.1% SDS at 65°C prior to exposure to

phosphorimaging plates. The plates are scanned using an ABI phosphorimager (Phosphorimager 445S1).

The method can also include performing *in situ* hybridization assays (ISH) to detect and quantify MK mRNA. A preferred method includes the following steps.

Sense and antisense digoxigenin-labeled (Boehringer Mannheim) riboprobes for human MK and fibroblast growth factor type 2 are transcribed *in vitro* for 2 hours at 42°C in the presence of 0.25 μM digoxigenin conjugated dUTP from 1 mg of linearized plasmid containing full-length cDNAs. Transcripts are treated with RNase-free DNase I, ethanol-precipitated, and resuspended in 100 mlDEPC-treated water.

ISH is essentially performed as described before (Panoskaltsis-Mortari and Bucy, 1995). Briefly, 10 μm thick tissue sections on silanized slides are washed, acetylated, and treated overnight at 55°C with hybridization buffer containing heat-denatured sense or antisense riboprobes. Sections are washed after hybridization at high stringency, immersed in blocking buffer, and immunohistochemistry is performed using an alkaline phosphatase-conjugated sheep-anti-digoxigenin antibody (1:500), following the manufacturer's instructions (Boehringer Mannheim). Chromogen substrate for alkaline phosphatase (NBT/BCIP) is added and the reaction is allowed to proceed overnight at 4°C. Color development is stopped with 10mM Tris-HCI, pH 8.0., 1 mM EDTA and tissue is mounted in an aqueous mounting medium.

In embodiments of the invention, the detection method detects abnormal levels of MK protein in blood serum of an individual. In an embodiment of the present invention, a method of detecting the MK growth factor protein in blood serum of an animal, especially a human, is provided. The blood serum can be obtained from any individual, including those suffering from NF1, those suspected of having NF1, those at high risk or having a predisposition for NF1, and even those with no apparent signs or predisposition for NF1. The novel method relies on the discovery that there is abnormal protein expression in the blood serum of NF1 patients.

In an embodiment of the invention, a neurofibromatosis type 1 disorder is diagnosed in an individual by a method comprising obtaining a blood serum sample from the individual; detecting the level of midkine protein in the serum sample; and determining whether the detected level of midkine protein is higher than a predetermined amount, the predetermined amount being greater than the concentration

of midkine protein in the blood serum of individual(s) that do not have a neurofibromatosis type 1 disorder. In this method, the predetermined amount may be at least three times higher than the concentration of midkine protein in the blood serum of individual(s) that do not have a neurofibromatosis type 1 disorder.

As used herein, the phrase "individual(s) that do not have a neurofibromatosis type 1 disorder" refers to a sample of at least one individual that does not have a neurofibromatosis type 1 disorder. Where the sample contains more than one such individual, the midkine protein level in the blood serum of the individual(s) is an average midkine protein level. The midkine protein level in each individual may be determined by testing one or more blood serum samples from each individual. Muramatsu et al. (J. Biochem, **119**:1171-1175, 1996) suggests an association between increased midkine levels in blood sera and hepatocellular carcinoma. Therefore, in selecting individuals that do not have a neurofibromatosis type 1 disorder, individuals that have this tumor and preferably individuals that have other tumors should not generally be selected.

In an embodiment of the invention, the predetermined amount is greater than or equal to one ng/mL of the serum.

The method can include obtaining a blood sample from the individual, e.g., peripheral blood, arm vein, etc., isolating the blood serum, detecting the level of MK growth factor protein in the blood serum, and comparing the detected level with the level of MK protein isolated from blood serum obtained from a normal (non-NF1) individual by the same technique, to determine if the MK protein is present in abnormal levels. This method not only provides a qualitative determination of the presence of MK at normal or abnormal levels, but also a semi-quantitative and quantitative method of determining the level of expression of the MK protein in the blood serum of the individual and a direct indication of the presence of the NF1 disorder in an individual.

Since increased levels of midkine protein in the blood serum may also be associated with other conditions, such as the presence of hepatocellular carcinoma, other tests to rule out these other conditions or to confirm the presence of a NF1 disorder, such as testing the skin of the individual, may also be needed to make a final diagnosis of NF1.

Techniques for isolating blood serum are well known to the skilled artisan. Any suitable technique can be used according to the present invention.

The method for isolation of the MK protein can include any protein purification method steps known to the skilled artisan that result in isolation and/or purification of the MK protein.

The method of detecting the MK protein can be any method for protein detection known to the skilled artisan, including staining for protein with a non-specific stain, such as coomassie blue staining of an acrylamide gel, specifically detecting the MK protein by using antibodies specific for the MK protein (e.g., Western blotting or other ELISA techniques), or determining absorbance at a wavelength of approximately 280 nm (UV absorption). In a particular embodiment of the present invention, the MK protein is detected by the technique described in Muramatsu et al. (J. Biochem, **119**:1171-1175, 1996), which discloses a highly sensitive enzyme-linked immunoassay for Midkine (MK) which involves affinity-purified anti-MK antibodies, their biotinylated form, and avidin-beta-galactosidase. The method allows the detection of human and mouse MK in the range of 50 pg to 10 ng.

In an embodiment, the invention provides a method of treatment of NF1. The method includes any of the above methods and further includes reducing the activity of MK in cells having abnormally high activity of the protein. The reduction step can be performed through any known technique for reducing protein activity, including, but not limited to, chemical methods which act to disrupt secondary or tertiary protein structure, interrupt protein-protein or protein-DNA interaction with the MK protein, MK-specific inhibition of the activity of MK through antibody-MK binding, and sequestering of the MK protein, such as in higher-order protein complexes.

In an embodiment, the invention provides a method of treatment of NF1 which includes any of the above methods and further includes reducing the level of MK in cells. The reduction step can be performed through any known technique for reducing protein levels, including, but not limited to, chemical methods which act to disrupt translation of mRNA to protein, specific inhibition of mRNA translation by specifically targeting the activity/level of ribosomes, and specific inhibition of translation of MKmRNA by binding complementary nucleic acids to the MKmRNA to block transcription (antisense).

In an embodiment, the present invention provides a method of treating NF1 which includes any of the above methods and further includes reducing the expression of MKmRNA. The reduction step can be performed through any known technique for reducing mRNA transcription, including, but not limited to, chemical methods which act to disrupt secondary or tertiary protein structure, interrupt protein-protein or protein-DNA interaction between DNA polymerase and/or transcription factors and DNA, inhibition of DNA polymerase activity, and antisense inhibition of expression of MKmRNA.

In preferred embodiments, the treatment methods of the invention are preferably used directly on the affected tumor cells. In particular, medicaments may be topically applied to the skin. When the treatment methods are applied directly to skin cells, a high level of an active agent, such as one that reduces MK protein levels, can be administered to the individual. This is because the active agent is directly applied to the site of action. Furthermore, by applying the method directly to skin cells, unwanted treatment of unaffected cells is minimized, thus improving the safety of the treatment method.

In preferred embodiments, the MK protein and mRNA are markers for NF1.

In preferred embodiments, the methods provide methods of diagnosis of NF1 because abnormal expression of MK growth factor protein and mRNA are indicative of NF1 gene mutation.

In a further embodiment, the present invention is directed to a method for diagnosing the presence of a tumor in an individual, particularly tumors other than carcinomas, more particularly tumors of the nervous system, such as neurofibromas, neurosarcomas, malignant peripheral nerve sheath tumors, gliomas, astrocytomas, rhabdomyosarcomas, and pheochromocytomas. In particular, it was found that individuals with NF1 that have a higher tumor burden generally have a higher level of midkine protein in their blood serum than individuals with NF1 that have a lower tumor burden. Thus, increased midkine levels in the blood serum of an individual appears to be associated with tumors, particularly tumors of the nervous system, in addition to being associated with the presence of a NF1 disorder.

Thus, the present invention is also directed to a method for diagnosing the presence of a tumor, particularly a tumor of the nervous system, comprising obtaining a blood serum sample from the individual; detecting the level of midkine protein in

the serum sample; and determining whether the detected level of midkine protein is higher than a predetermined amount, the predetermined amount being greater than the concentration of midkine protein in the blood serum of individual(s) that do not have a tumor. In this method, the predetermined amount may be at least three times higher than the concentration of midkine protein in the blood serum of individual(s) that do not have a tumor.

The blood serum can be obtained from any individual, including those having a tumor, particularly of the nervous system; those suspected of having a tumor, particularly of the nervous system; those at high risk or having a predisposition for a tumor, particularly of the nervous system, such as those having a prior medical history or a known family history therefor; and even those with no apparent signs or predisposition for tumors, particularly of the nervous system.

As used herein, the phrase "individual(s) that do not have a tumor" refers to a sample of at least one individual that does not have a tumor. Where the sample contains more than one such individual, the midkine protein level in the blood serum of the individual(s) is an average midkine protein level. The midkine protein level in each individual may be determined by testing one or more blood serum samples from each individual. As discussed above, there is also an association between increased midkine levels in blood sera and NF1. Therefore, in selecting individuals that do not have a tumor, individuals that have NF1 should not generally be selected.

In an embodiment of the invention, the predetermined amount is greater than or equal to one ng/mL of the serum.

Since increased levels of midkine protein in the blood serum may also be associated with other conditions, other tests to rule out these other conditions or to confirm the presence of a tumor may also be needed to make a final diagnosis of a tumor and to determine where the tumor is. For example, an MRI may be used to confirm the initial diagnosis and/or to locate the tumor.

In a further embodiment, the present invention provides a method of culturing tissue culture cells by adding purified MK to the tissue culture medium. The medium so created shows enhanced growth of tissue culture cells and provides an improved method of culturing cells, including cells that could not be cultured previously. The present invention discloses that MK acts as a mitogen for both endothelial cells and neurofibroma-derived cells of fibroblast morphology. Thus, its expression by

keratinocytes can support the growth of the various cell types comprising neurofibromas, including fibroblasts and vascular elements.

EXAMPLES

Tissues and cell lines: Neurofibroma and normal skin samples were obtained from the Lombardi Cancer Center tissue facility. The cell line Hs422T of fibroblast morphology is derived from the neurofibroma of an NF1 patient and was obtained from American tissue type Culture Collection (ATCC, Rockville, MD). Human umbilical vein endothelial cells (HUVEC) were obtained from the Lombardi Cancer Center cell culture core. SW-13, SW-13 transfected with an empty pRC/CMV control vector, and SW-13 transfected with pRC/CMV-human MK expression construct (MK6) obtained from the Lombardi Cancer Center.

Proliferation Assays: Purified MK was used at a concentration of approximately 8ng/ml for proliferation assays. Cell lines used to collect conditioned media were SW13 (adrenal carcinoma), SW-13 cells transfected with the pRC/CMV control vector, and an SW13 cell line overexpressing human recombinant MK from an MK-pRC/CMV expression construct (termed MK6). Cells were cultured to confluence in a T75 flask, and growth media was replaced with 5 mlserum free media (IMEM) that was collected 24 hours later. Media was used directly after centrifugation and filtration to remove cellular elements. HUVEC and Hs422T neurofibroma derived cells, respectively, were plated at 1000 cells/well in a fibronectin coated 96-well plate in IMEM/10% FBS. After 16 hours, 100 mlof serum free media, SW13, or MK6 conditioned media were added in triplicate. At day four after stimulation, the media were replaced with 100 mlIMEM/10% FBS + 10 mlWST-1 colorimetric reagent (Boehringer Mannheim). At 30 minutes after addition of reagent, plates were analyzed at a reference 1 of 600 nm and test 1 of 450 nm.

Example 1: Midkine is Differentially Expressed in the Skin of NF1 Patients

Neurofibromin is expressed in Schwann cells (Nakamura T. et al., 1994), and its loss in these cells results in the upregulation of MK1. Because neurofibromin is also expressed in keratinocytes and melanocytes (Malhotra and Ratner, 1994; Hermonen et al., 1995), and because the symptoms of NF1 are predominantly cutaneous, the levels of MK are assayed to determine whether MK is abnormally upregulated in the skin of NF1 patients. *In situ* hybridization (ISH) reveal dramatic expression of MK transcript in the keratinocytes of NF1 patients. MKmRNA is

detected in the skin of NF1 patients in all layers of epidermis overlying neurofibromas (n=4) (Fig. 1a), and is also found in cells of dermal neurofibromas (not shown). Importantly, skin over a solitary neurofibroma from a non-NF1 subject demonstrates little to no MK expression (n=1) (Fig. 1b), suggesting that expression is particular to NF1 patients. Skin without phenotypic abnormality from an NF1 patient is also found to be positive for MK (n=1) (Fig. 1c), while normal skin from healthy subjects is negative for MK expression by ISH (n=4) (Fig. 1d) and Northern analysis (n=6) (not shown). No signal is detected using a MK sense riboprobe (not shown). These data show that the induction of MK expression in neurofibromin-deficient keratinocytes is linked to the NF1 mutation itself, and not simply to the presence of an underlying neurofibroma.

MK mRNA expression in the keratinocytes of NF1 patients is paralleled by an identical pattern of MK protein expression, as shown by immunohistochemistry (n=3 for NF1 skin over a neurofibroma, n=3 for NF1 skin without phenotypic abnormality) (Fig. 1e). Although MK mRNA is not detected in the dermis of NF1 skin without phenotypic abnormality, MK protein did appear to be present. MK is a secreted protein, and has previously been detected in areas where transcript is not expressed (Muramatsu et al., 1993). In contrast, the epidermal and dermal layers of normal human skin are MK-negative by immunohistochemistry (n=3) (Fig. 1f), as was previously demonstrated (Inazumi et al., 1997). We attribute staining of the stratum corneum to nonspecific antibody binding, as this is found in samples which demonstrate no MK mRNA expression. No MK protein is detected in skin from non-NF1 neurofibromas (n=2) (not shown). These results are summarized in Table 1.

Homozygous loss of NF1 in Schwann cells results in aberrant angiogenic activity and a shift of angiogenic factor expression, including upregulation of MK, fibroblast growth factor-2 (FGF-2), and others. In order to determine whether loss of neurofibromin in skin results in the same shift of angiogenic profile, NF1 and non-NF1 skin were studied for expression of FGF-2. Skin overlying a neurofibroma from an NF1 patient revealed FGF-2 mRNA expression in the dermal layer, in blood vessels, and sebaceous glands (n=3) (Fig. 1g). This is the expected distribution of FGF-2, and indeed there was no difference when compared to normal skin from a healthy subject (n=3) (Fig. 1h). Thus, the shift of the angiogenic profile in

neurofibromin-deficient skin does not reflect the shift in neurofibromin-deficient Schwann cells.

Table 1: MK Expression in NF1 and non-NF1 Skin.

This table summarizes detection of MK in various samples using the techniques of ISH, Northern analysis, and immunohistochemistry, and is expressed as MK-positive samples/samples analyzed. N.D. = not determined.

Sample	*In situ* Hybridization	Northern Analysis	Immunohisto-chemistry
NF1 epidermis over neurofibroma	4/4	n.d.	3/3
NF1 epidermis without phenotypic abnormality	1/1	n.d.	3/3
Non-NF1 epidermis over neurofibroma	0/1	n.d.	0/2
Normal Skin	0/4	0/6	0/3

Example 2: Midkine Protein is Present at Abnormally High Levels in the Blood Serum of NF1 Patients

Blood (5-15 mL) taken from subjects is allowed to clot at room temperature and the resulting serum is used for the assay. The serum is diluted twofold in phosphate buffered saline and applied to a heparin sepharose column. The column is washed with 10 mL of 0.5 M NaCl. MK is eluted from the heparin sepharose in 0.5 mL of 1.5 M NaCl.

For the ELISA (enzyme linked immunosorbent assay), 100 µL of the 1.5 M eluate is added per well of a 96-well plate and the plate is incubated at 37°C for 1 hour. Next, the wells are washed 4 times in water. Next, 100 µL blocking buffer (0.25% bovine serum albumin, 0.05% Tween 20, 0.1 M EDTA) is added to the wells and incubated for 1 hour at room temperature. Next, the wells are washed 3 times in water. Next 100 µL anti-MK antibodies made in goat (R&D Systems, Minneapolis, MN) at 1:500 diluation are added to the wells and incubated for 2 hour at room temperature. Next, the wells are washed 4 times in water. Next, 100 µL biotinylated anti-goat antibodies (Vector Labs, Burlington, CA) at 1:1000 are added to the wells

and incubated for 2 hours at room temperature. Next, the wells are washed 4 times in water. Next, 100 µL streptavidin coupled to horseradish peroxidase (Zymed, San Francisco, CA) at 1:4000 is added to the wells and incubated for 1 hour at room temperature. Next, the wells are washed 4 times in water. Next, 100 µl 3'3'5'5'-tetramethylbenzidine substrate solution (Kirkegaard & Perry, Gaithersburg, MD) is added to the wells and incubated for 30 minutes at room temperature.

Light absorption/fluorescence is measured at 650 nm. Data from NF1 patients (n=6) are plotted against data from normal subjects (n=3). The results are depicted in Fig. 2. The increase of MK levels in NF1 patients is at least 2-fold compared to normal controls.

The NF1 patents tested had dermal neurofibromas. Of the NF1 patients, patients without gliomas (n=4) had a MK level of greater than 2000 fluorescence units, which indicates a midkine concentration of greater than 1 ng/mL. NF1 patients with gliomas (n=2) had a MK level of greater than 6000 fluorescence units, indicating higher MK levels. These results are depicted in Fig. 2, with the samples between 6000 and 8000 representing samples with gliomas.

Example 3: MK is a Mitogen for Human Endothelial and Neurofibroma-derived Cells

Human umbilical vein endothelial cells (HUVEC) and Hs422T neurofibroma-derived cells were plated at 1000 cells/well in a fibronectin coated 96-well plate in IMEM/10% FBS. After 16 hours, 100 ml of serum free media, SW13, or MK6 conditioned media were added in triplicate. At day four after stimulation, the media were replaced with 100 ml IMEM/10% FBS + 10 ml WST-1 colorimetric reagent. At 30 minutes after addition of reagent, plates were analyzed at a reference 1 of 600 nm and test 1 of 450 nm.

To test whether MK could contribute to neurofibroma tumorigenesis and angiogenesis, conditioned media from MK6 cells overexpressing human MK is used. The MK6 cell line was generated from the MK-negative adrenal carcinoma cell line SW-13. Conditioned media from MK6 cells induces a 263.84% (mean, SD + .23%) stimulation of human umbilical vein endothelial cells growth compared to media from the parent cell line SW-13 (Fig. 3). In order to confirm that the mitogenic effects were attributable to MK, the conditioned media of MK6 cells was partially purified using heparin-affinity chromatography. Since the 0.9 M NaCl eluate contains MK,

this fraction was used in an endothelial cell proliferation assay. Using this partially purified conditioned media from MK6 cells, a dose-dependent stimulation of HUVEC proliferation was found that was not found with control media conditioned by SW13 cells or a control-transfected SW13 clonal cell line (not shown). Conditioned media from MK6 cells demonstrated a mitogenic effect on the neurofibroma-derived fibroblast cell line Hs422T. MK6 conditioned media induced a 197.53% (mean, SD + 3.36%) stimulation of Hs422T cells compared with SW13 conditioned media (Fig. 3).

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WHAT IS CLAIMED IS:

1. A method for diagnosing and/or treating a neurofibromatosis type 1 disorder in an individual, comprising:

obtaining a skin sample from the individual; and

detecting the presence of midkine protein and/or the mRNA sequence encoding said midkine protein in said skin sample, the presence of a detectable amount of said midkine protein and/or said mRNA sequence in the sample indicating the likelihood of a neurofibromatosis type 1 disorder.

2. The method of claim 1, wherein the presence of said mRNA is detected.

3. The method of claim 1, wherein the presence of said protein is detected.

4. The method of claim 3, wherein the presence of an amount of midkine protein greater than or equal to 10 ng/mm^3 of skin indicates the likelihood of a neurofibromatosis type 1 disorder.

5. The method of claim 1, wherein said skin sample is obtained through a skin biopsy.

6. The method of claim 5, wherein said skin sample is stained to detect the midkine protein and/or the mRNA sequence encoding said midkine protein.

7. The method of claim 1, wherein said individual is suspected of having a neurofibromatosis type 1 disorder.

8. The method of claim 1, wherein said individual is predisposed to having a neurofibromatosis type 1 disorder.

9. The method of claim 1, said method further comprising reducing the activity or level of midkine protein in cells of the individual.

10. A method for diagnosing and/or treating a neurofibromatosis type 1 disorder in an individual, comprising:

obtaining a skin or blood serum sample from the individual;

detecting the level of midkine protein present in the sample; and

comparing the detected level of midkine protein in said sample to the level of midkine protein present in at least one corresponding skin or blood serum sample of at least one individual that does not have a neurofibromatosis type 1 disorder to determine if it is likely that the individual being tested has a

neurofibromatosis type 1 disorder, wherein a higher level of midkine protein in the sample of said individual being tested as compared to in the at least one corresponding sample of said at least one individual that does not have a neurofibromatosis type 1 disorder indicates the likelihood of a neurofibromatosis type 1 disorder in said individual being tested.

11. The method of claim 10, wherein said sample is a skin sample.

12. The method of claim 10, wherein said sample is a blood serum sample.

13. The method of claim 10, wherein said individual is suspected of having a neurofibromatosis type 1 disorder.

14. The method of claim 10, wherein said individual is predisposed to having a neurofibromatosis type 1 disorder.

15. The method of claim 10, further comprising conducting a further test to confirm a diagnosis of a neurofibromatosis type 1 disorder.

16. The method of claim 15, wherein said further test comprises detecting the presence of midkine protein and/or the mRNA sequence encoding said midkine protein in a skin sample of the individual.

17. The method of claim 10, said method further comprising reducing the activity or level of midkine protein in cells of the individual.

18. A method for diagnosing and/or treating a neurofibromatosis type 1 disorder in an individual, comprising:

obtaining a skin or blood serum sample from the individual;

detecting the level of midkine protein in said sample; and

comparing said detected level of midkine protein to a predetermined amount to determine if it is likely that the individual has a neurofibromatosis type 1 disorder, said predetermined amount being greater than the concentration of midkine protein in the skin or blood serum of individual(s) that do not have a neurofibromatosis type 1 disorder, wherein the presence of an amount higher than the predetermined amount indicates the likelihood of a neurofibromatosis type 1 disorder.

19. The method of claim 18, wherein said sample is a skin sample.

20. The method of claim 19, wherein said predetermined amount is greater than or equal to 10 ng/mm^3 of skin.

21. The method of claim 18, wherein said sample is a blood serum sample.

22. The method of claim 21, wherein said predetermined amount is at least three times higher than the concentration of midkine protein in the blood serum of said individual(s) that do not have a neurofibromatosis type 1 disorder.

23. The method of claim 21, wherein said predetermined amount is greater than or equal to one ng/mL of serum.

24. The method of claim 18, wherein said individual is suspected of having a neurofibromatosis type 1 disorder.

25. The method of claim 18, wherein said individual is predisposed to having a neurofibromatosis type 1 disorder.

26. The method of claim 18, further comprising conducting a further test to confirm a diagnosis of a neurofibromatosis type 1 disorder.

27. The method of claim 26, wherein said further test comprises detecting the presence of midkine protein and/or the mRNA sequence encoding said midkine protein in a skin sample of the individual.

28. The method of claim 18, said method further comprising reducing the activity or level of midkine protein in cells of the individual.

29. A method for diagnosing the presence of a tumor of the nervous system in an individual, comprising:

obtaining a blood serum sample from the individual;

detecting the level of midkine protein present in the sample; and

comparing the detected level of midkine protein in said sample to the level of midkine protein present in at least one blood serum sample of at least one individual that does not have a tumor to determine if it is likely that the individual being tested has a tumor of the nervous system, wherein a higher level of midkine protein in the sample of said individual being tested as compared to in the at least one blood serum sample of said at least one individual that does not have a tumor indicates the likelihood of the presence of a tumor of nervous system in said individual being tested.

30. The method of claim 29, wherein said individual is suspected of having a tumor of the nervous system.

31. The method of claim 29, wherein said individual is predisposed to having a tumor of the nervous system.

32. A method for diagnosing the presence of a tumor of the nervous system in an individual, comprising:

obtaining a blood serum sample from the individual;

detecting the level of midkine protein in said sample; and

comparing said detected level of midkine protein to a predetermined amount to determine if it is likely that the individual has a tumor of the nervous system, said predetermined amount being greater than the concentration of midkine protein in the blood serum of individual(s) that do not have a tumor, wherein the presence of an amount higher than the predetermined amount indicates the likelihood of the presence of a tumor of the nervous system.

33. The method of claim 32, wherein said predetermined amount is at least three times higher than the concentration of midkine protein in the blood serum of said individual(s) that do not have a tumor.

34. The method of claim 32, wherein said predetermined amount is greater than or equal to 1 ng/mL of serum.

35. The method of claim 32, wherein said individual is suspected of having a tumor of the nervous system.

36. The method of claim 32, wherein said individual is predisposed to having a tumor of the nervous system.

37. A method for culturing tissue cells, comprising:

adding a midkine protein to a culture medium containing said tissue cells; and

culturing said tissue cells in said medium.

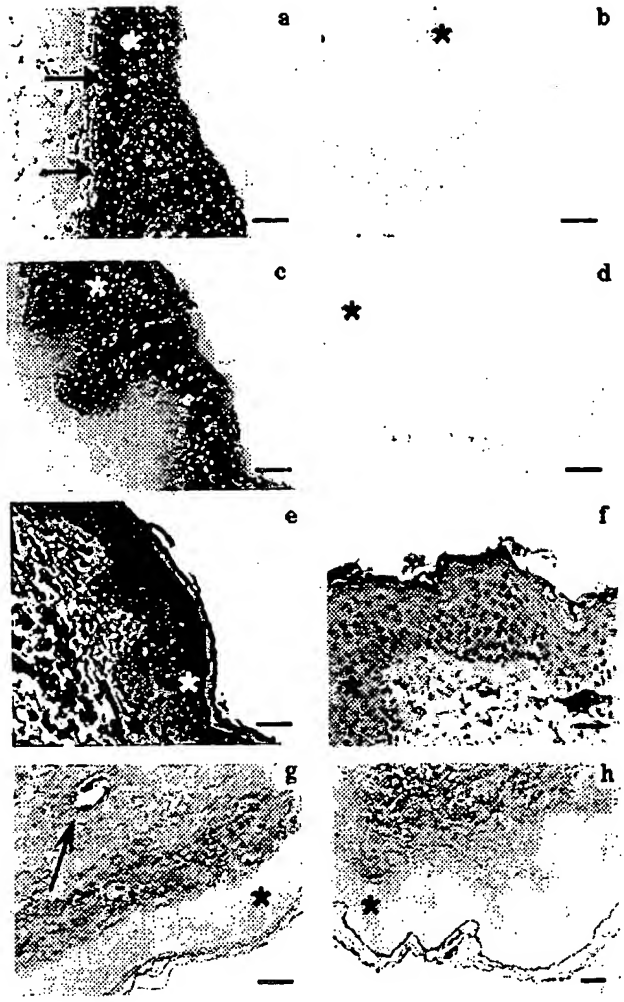


Figure 1

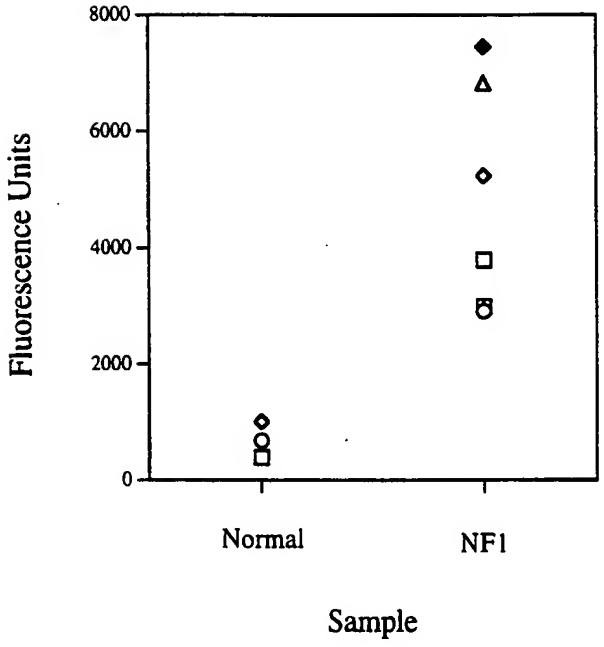
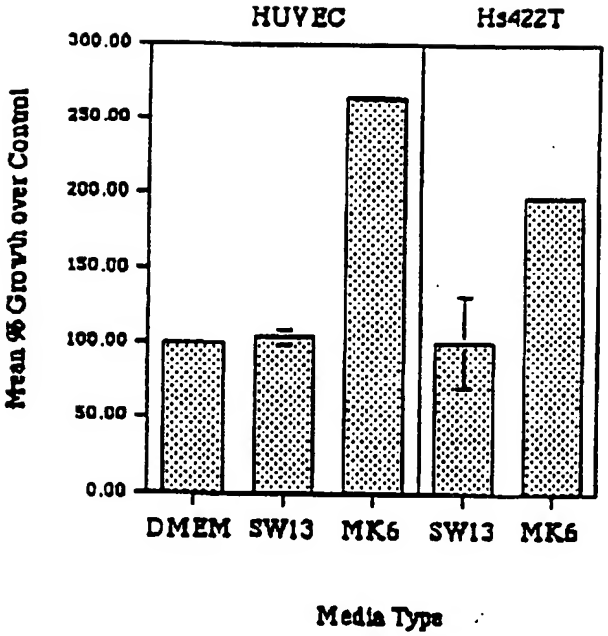


Figure 2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Mean % Growth over Control
DMEM
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SW13
MK6
Media Type

Figure 3

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(57) Abstract

Neurofibromatosis type 1 disorders result in abnormal levels of midkine protein and/or the mRNA sequence encoding the midkine protein in the skin and/or blood serum of individuals having the disorder. Thus, test for abnormal levels of midkine protein and/or the mRNA sequence that encodes the midkine protein can be used to diagnose a neurofibromatosis type 1 disorder.

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DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
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INTERNATIONAL SEARCH REPORT

	International Application No PCT/US 99/27292

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/68 G01N33/574 C12N5/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIOLOGICAL ABSTRACTS, Philadelphia PA USA; abstract no. PREV199799766263, abstract XP002136900 & G.A. MASHOUR ET AL.: "Midkine is differentially expressed in neurofibromin-deficient Schwann cells and human neurofibromas " SOCIETY FOR NEUROSCIENCE ABSTRACTS , vol. 23, no. 1-2, 25 October 1997 (1997-10-25), page 88 New Orleans, Louisiana, USA	1-37

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[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
3 May 2000	24/05/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Van Bohemen, C

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INTERNATIONAL SEARCH REPORT

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	PCT/US 99/27292

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X,P	MEDLINE, Washington DC USA; abstract no. 99398478, abstract XP002136901 & G.A. MASHOUR ET AL.: "Aberrant cutaneous expression of the angiogenic factor midkine is associated with neurofibromatosis type-1 " JOURNAL OF INVESTIGATIVE DERMATOLOGY, vol. 113, no. 3, 1 September 1999 (1999-09-01), pages 398-402, Washington DC USA	1-37
X	CHEMICAL ABSTRACTS, vol. 119, no. 23, 6 December 1993 (1993-12-06) Columbus, Ohio, US; abstract no. 242025, XP002136902 abstract & S. KIKUCHI ET AL.: "Midkine , a novel neurotrophic factor, promotes survival of mesencephalic neurons in culture " NEUROSCIENCE LETTERS , vol. 160, no. 1, 1993, pages 9-12, Amsterdam NL	37

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